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Our purpose is to characterize the proposed p53 regulatory domain and identify kinase(s) that may be involved in the regulation. Since 7/1/97, we have constructed a set of Gal4-p53N mutations and assayed their transcription activity in vivo. Our preliminary data show that stimulation by TPA reduced transcriptional activity of Gal4-p53N(Δ92-109) and Gal4-p53N(Δ117-128), but not Gal4-p53(1-92), suggesting that residues 92-109 and 117-128 indeed play a role to reduce p53 transcription activity in response to TPA. In addition, we also show that co-transfection with MAPK but not mutant MAPK reduced p53 transcription activity, suggesting a role of MAPK in regulation of p53 transcription activity.

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Introduction

A putative domain may be present within p53 involving its regulation (Liu et al., 1993). Further evidence of a regulatory domain is derived from a comparison of p53 with two oncogenes, c-jun and c-fos. Amino acids 93-160 from p53 show a significant homology to c-jun and c-fos inhibitory domains, especially to c-jun's δ region (Baichwal and Tjian, 1990). The c-jun δ region has been found to play an inhibitory role by interacting with a member of the MAPK family, JNK (Kyriakis et al., 1994). Once bound to c-jun, JNK is capable of phosphorylating serine residues 63 and 73 (Derijard et al., 1994). Two conserved serine residues within p53's potential regulatory domain may also be involved in phosphorylation and regulation. Therefore, we hypothesized that p53 serine residues 117 and 127, homologous to c-jun serine residues 63 and 73, may be phopshorylated by JNK or another MAPK family member. Specifically, we proposed:
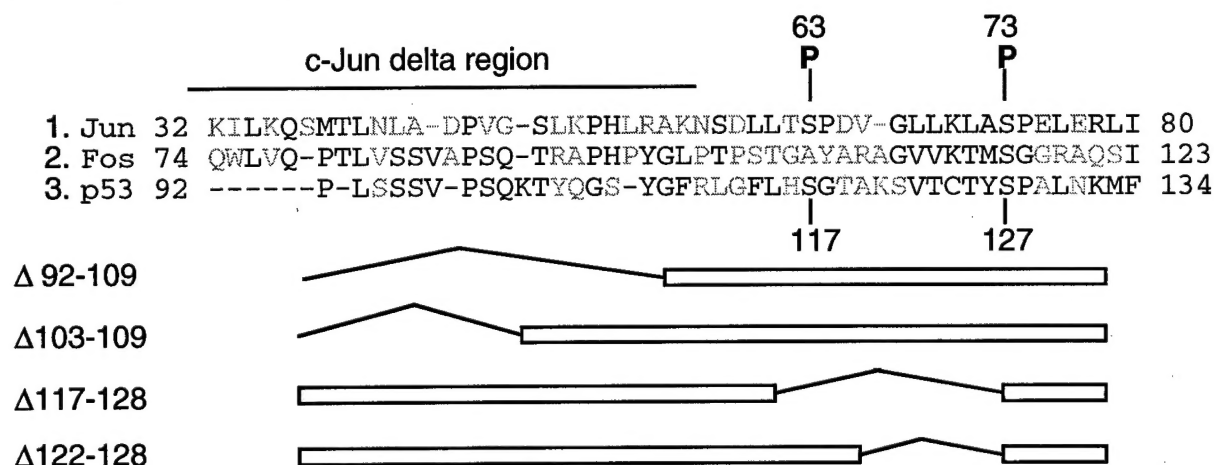
I.   Construct Gal4-p53(Δ92-109) and Gal4-p53(Δ117-128) and study their transcription activities using a transient transfection assay.

II.   Assay if JNK is involved in regulating p53 transcription activity by co-transfecting with JNK.

III.   Construct point mutants on serine residues 117 and 127.

IV.   Study phosphorylation of p53 regulatory domain by JNK or other potential kinase(s).

Background

p53 is an important tumor suppressor gene, mutated, or absent, in over 50% of all cancers studied (Ko and prives, 1996; Levine, 1997). It functions as a sequence-specific DNA-binding transcription factor. In response to double-stranded DNA breaks p53 is converted from a latent to an active form. This results in increased expression of p53-responsive proteins such as p21 which are required for growth arrest at the G1 to S phase transition. It also mediates apoptosis via the increased expression of proteins such as Bax (Miyashita and Reed, 1995). Inactivation of p53, therefore, results in the loss of a cell cycle checkpoint required for repair of damaged DNA and prevents apoptosis in response to severe DNA damage. In the absence of these responses oncogenic mutations can accumulate which may result in tumor progression. From the above, it

is clear that the transcriptional activation function of p53 is critical to its role as a tumor suppressor.

Previous work by our group suggests a possible regulatory domain may be present within p53. Supporting preliminary data used Gal4-p53 constructs for deletion analysis of p53's transcription activity. Based on transient transfection assays, the region of p53 spanning residues 1-92 shows high transactivation activity. Extension of the transcriptionally active residues 1-92 with residues 93 to 160 resulted in a reduction in transcription by a factor of 100. Similar results have been observed in the c-jun and c-fos proteins, both of which contain conserved inhibitory domains capable of silencing their activation domains. Furthermore, the δ region of the c-jun inhibitory domain has been shown to interact with the c-jun N-terminal kinase (JNK; Kyriakis et al., 1994). JNK is a member of the mitogenic activated protein kinase (MAPK) family which can positively regulate c-jun activity through phosphorylation of serine residues 63 and 73 (Derijard et al., 1994). A comparison of the regulatory regions of c-jun and c-fos has identified homology with a p53 inhibitory domain (92-160). It is interesting to note that the proposed p53 regulatory region also contains two conserved serine residues at position 117 and 127 which may also be phosphorylated by JNK or a JNK related kinase. Therefore, we proposed to characterize this putative p53 inhibitory domain.


```
                                                    63          73
                                                    P           P
              c-Jun delta region                    |           |
1. Jun 32 KILKQSMTLNLA-DPVG-SLKPHLRAKNSDLLTSPDV-GLLKLASPELERLI 80
2. Fos 74 QWLVQ-PTLVSSVAPSQ-TRAPHPYGLPTPSTGAYARAGVVKTMSGGRAQSI 123
3. p53 92 ------P-LSSSV-PSQKTYQGS-YGFRLGFLHSGTAKSVTCTYSPALNKMF 134
                                                |           |
                                               117         127
```

Δ 92-109

Δ103-109

Δ117-128

Δ122-128

Experimental Methods and Procedures

Construction of mutants

Deletion mutants pGal4-p53(Δ92-109), pGal4-p53(Δ117-128) and pGal4-p53(Ser117/127Ala) were constructed by PCR mutagenesis using the plasmid pGal4-p53N

which contains Gal4-p53(1-160) (Liu et al., 1993) as a template and oligonucleotides which introduce mutations at corresponding positions. All the mutants were confirmed with DNA sequencing. For transient transfection assay, a fragment containing 5 Gal4 binding sites and Ad E4 TATA box was cut from pG5E4CAT (Carey et al., 1990) and cloned into pZluc (provided by F. Sladek) between KpnI and SmaI sites, creating, pG5E4Luc.

Cell culture and transient transfection

SAO-S2 cells were cultured in DMEM media supplemented with 10% FBS. The cells were plated at 2×10^6 cells per 60-mm plate and transfected by the calcium phosphate method with 1 μg of pG5E4Luc, 1 μg of the β-galactosidase expression plasmid pCH110, and 0.2 μg of effector DNA (pGal4-p53N constructs). Cells were harvested 36 hr post-transfection. All samples were normalized for transfection efficiency by measuring β-galactosidase activity. Luciferase activity was measured by use of the Lumat Luminometer with the Promega Luciferase Assay Kit. β-galactosidase activity was assayed with the Tropix Galacton Assay System.

Results and Discussion

We have successfully completed Aims 1 and 2 and obtained following results:

1. **Mutants Gal4-p53N(Δ92-109), Gal4-p53N(Δ117-128) and Gal4-p53N(Ser117/127Ala) had little effect on the putative inhibitory effect.** To study the putative inhibitory domain, we constructed a set of mutants, Gal4-p53N(Δ92-109), Gal4-p53N(Δ117-128) and Gal4-p53N(Ser117/127Ala), and examined their ability to activate transcription in vivo. This was done by using a transient transfection assay in COS-7 cells in which Gal4-p53 fusion proteins are tested for their ability to stimulate expression of luciferase gene under control of a promoter containing five Gal4 binding sites upstream of a TATA box. Gal4-p53N and Gal4-p53(1-92) were used as controls. Results of representative luciferase assay are shown in Figure 1. This result reveals that low levels of transcriptional activity of the mutant Gal4-p53 which are similar to Gal4-p53N, suggesting that mutants Gal4-p53N(Δ92-109), Gal4-

p53N(Δ117-128) and Gal4-p53N(Ser117/127Ala) had little effect on the putative inhibitory effect.
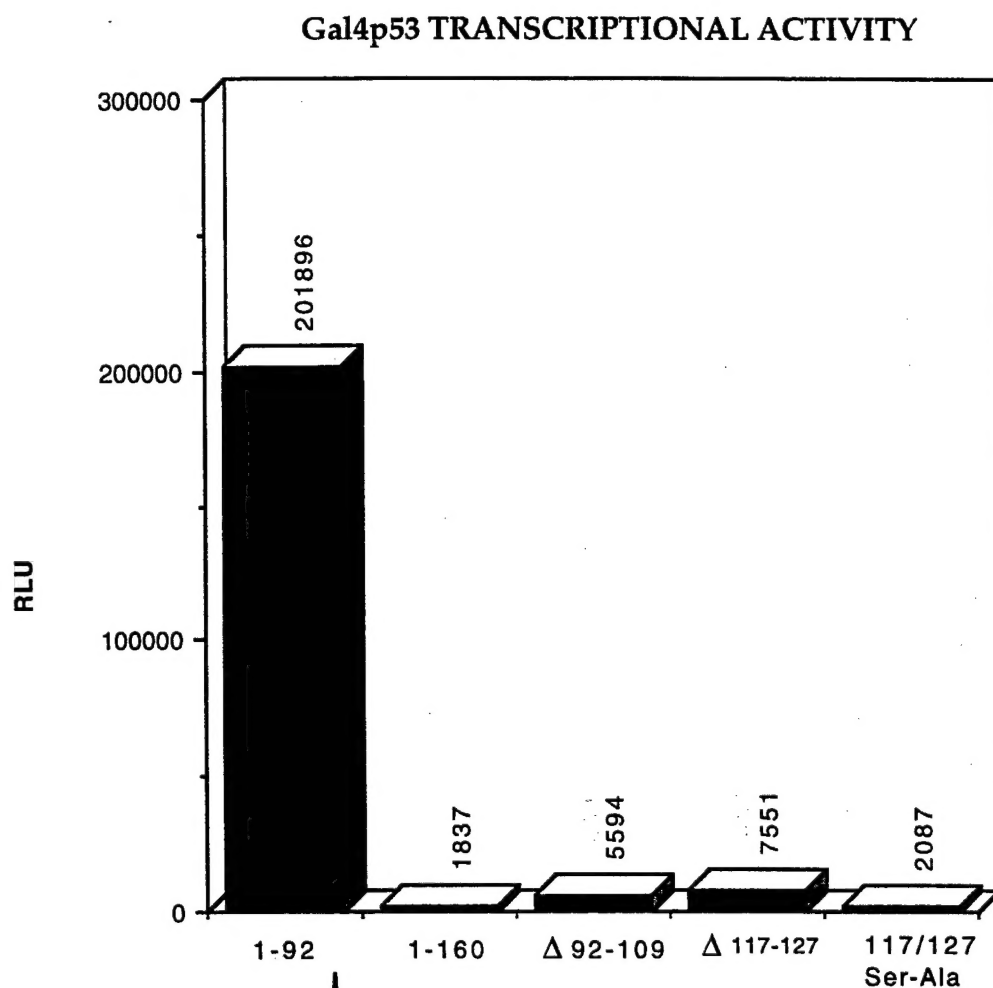
Gal4p53 TRANSCRIPTIONAL ACTIVITY

Fig 8. Transcriptional activity of Gal4p53 constructs measured using a Luciferase reporter plasmid in transfected COS-7 cells.

2. **TPA stimulation inhibited the transcriptional activities of Gal4-p53N(Δ92-109) and Gal4-p53N(Δ117-128), but did not affect Gal4-p53(1-92).** We consider the possibility that failure to detect any effect for the mutants may be caused by a lack of JNK or JNK related kinase activity in the cells. To test this, JNK activity was stimulated by TPA. This was done by treating NIH3T3 cells with TPA at different times after transfection. Results of representative luciferase assay are shown in Table I. These results show that stimulation by TPA reduced transcriptional activity of Gal4-p53N(Δ92-109) and Gal4-p53N(Δ117-128), but did not affect Gal4-p53(1-92), suggesting that residues 92-109 and 117-128 indeed play a role to reduce p53 transcription activity in response to TPA.

3. **MAPK is involved in regulating p53 transcription activity.** TPA has been shown to stimulate JNK and several other kinases including MAPK. To assay if JNK is involved in this regulation, plasmids expressing JNK or JNK dominate negative mutant was co-transfected with the Gal4-53N fusion constructs respectively. Our result show that JNK had little effect on the transcription activity of the Gal4-53 fusion constructs (Figure 2). Using a similar strategy we next tested if MAPK is involved in the regulation (Figure 2) and our results show that co-transfection with MAPK but not MAPK mutant indeed reduced p53 transcription activity, suggesting a role of MAPK in regulation of p53 transcription activity.
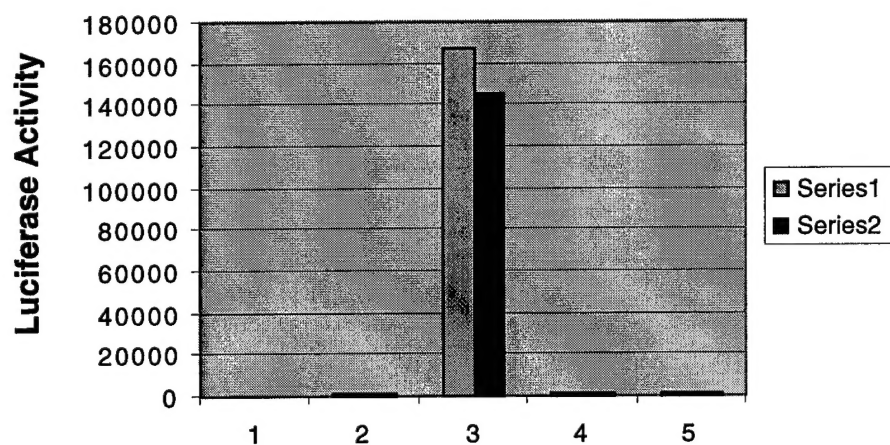
Comparison of Different Cell Types Using Transient Transfection Luciferase Data

	3T3 "Swiss" Trial #1	3T3 "Swiss" Trial #2	3T3L1 "Fat" No Stimulation	3T3L1 "Fat" TPA Stimulated
E4 Luc	0	0	136	0
Gal4p53N	0	0	0	0
Gal4p53(1-92)	0	51	4236	5679
Gal4p53(1-160)Δ92-109	0	0	906	388
Gal4p53(1-160)Δ117-128	0	0	663	184
Gal4VP16	1008	0	83360	127590

A.
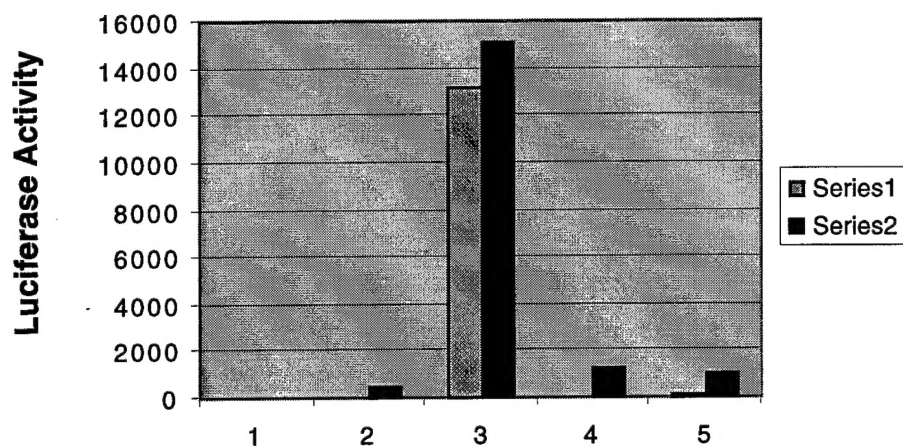
B.

Figure 2 Effects of JNK and MAPK on p53 transcription. A. MAPK (series 1) or mutant MAPK (series 2) was cotransfected with Gal4-p53 constructs: 1, reporter alone; 2, Gal4-p53N; 3, Gal4-p53(1-92); 4, Gal4-p53N(Δ92-109) and 5, Gal4-p53N(Δ117-128). B JNK (series 1) or mutant JNK (series 2) was cotransfected with Gal4-p53 constructs as above.

Recommendation in Relation to the Statement of Work

Proposed research has been accomplished according to SOW. Specifically:

1. Construct Gal4-p53(Δ92-109) and Gal4-p53(Δ117-128) and study their transcription activities using a transient transfection assay. **Completed.**

2. Assay if JNK is involved in regulating p53 transcription activity by co-transfecting with JNK. **In progress.**

3. Construct point mutants on serine residues 117 and 127. **Completed.**

Conclusions

We have demonstrated that residues 92-109 and 117-128 indeed play a role to reduce p53 transcription activity and MAPK is involved in this reduction. To test the significance of data, we will focus our effects on obtaining mutants on full-length p53 background.

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